Proteomic analysis of important brain areas in the regulation of maternal behaviour in rats

PhD Theses

Edina Brigitta Udvari

Eötvös Loránd University, Faculty of Science

Biology Doctoral School

Neuroscience and Human Biology Program

Head of Doctoral School: Dr. Anna Erdei, PhD, DSc

Programme leader: Dr. László Détári, PhD, DSc

Supervisors: Dr. Árpád Dobolyi, PhD, DSc

Department of Physiology and Neurobiology, MTA-ELTE Laboratory of Molecular and Systems Neurobiology

Dr. Katalin Adrienna Kékesi, PhD

Department of Physiology and Neurobiology, Laboratory of Proteomics

EÖTVÖS LORÁND UNIVERSITY

FACULTY OF SCIENCE

Biology Doctoral School, Laboratory of Proteomics, MTA-ELTE Laboratory of Molecular and Systems Neurobiology

Budapest, 2019

Introduction

The parental care of the offspring is a special form of social behaviours (Chen et al., 2018). Its regulation is based on fine-tuning of the brain regions also involved in the control of social behaviours (Goodson 2005). That fine-tuning mechanism is the neurobiological background of the behavioural level differences between caring behaviour and other social behaviours. Thus, research of the molecular mechanisms can lead to breakthroughs in the understanding of the brain mechanisms responsible for parental behaviours. However, these molecular mechanisms have not been recognized yet.

The functional connectome network of the brain regions typically contains some richly connected hubs and less connected parts. As my working hypothesis I assumed that a widespread adjustment of brain social behaviour network in mothers underlying parental care is performed by fine-tuning of connectome hubs, which can in turn effectively alter the behavioural output of the whole network due to their high density connectedness. The hypothalamus, the preoptic area (POA) and the medial prefrontal cortex (mPFC) are such a hub regions in the social brain network (Saper et al., 2014, Dulac et al., 2014, Dalley et al., 2004). Therefore, my experiments were carried out in these selected brain regions.

During pregnancy and in the *postpartum* period there are various changes in the hormonal system, which can induce changes in the transcriptome, and also in the proteome level via transcription regulation mechanism (Gammie et al., 2016, Driessen et al., 2014, Eisinger et al., 2014). Proteins perform the functional operations in the cells, which makes the proteome more relevant functionally than the transcriptome. Still, only transcriptomics studies have been performed regarding the parental brain.

The most important functions of the neurons are information processing and transmission through synapses, which determine the strength of a neuron and thus its role in the network. The molecular mechanisms of the synaptic strength are an intensively examined field, particularly the molecular mechanisms of long-term potentiation. However, the permanent synaptic fine-tuning mechanisms are mostly unknown. The synaptic plasticity lead to dynamic changes in the neuron, which can indirectly affect different levels of the cellular processes (e.g. metabolic alterations, cytoskeleton reorganization). In the selected brain areas, these kinds of plastic proteome alterations may be behind the regulation of the behavioural processes, which should be addressed. However, the identification of the entire proteome has some technical limitations. Due to the absence of protein amplification methods, only 10-15% of the proteome can be detected. Therefore, the revealed protein changes give only some

limited information about the most abundant proteins while the less abundant ones remain hidden. Bioinformatical modelling of protein networks is capable to extend our knowledge. Still, we can make only experimentally testable data-based working hypotheses from the proteomic results.

Aims of the study

The primary purpose of my study was to reveal the protein level alterations with proteomics techniques in the three selected brain areas (hypothalamus, POA, mPFC) during the *postpartum* period. Using bioinformatics, we also addressed the role of hormonal system in the regulation of the protein level changes. Our further aim was to validate some outstanding protein level changes with independent methods and analyse the spatial distribution of these proteins in the examined brain regions. Therefore, our specific objectives were as follows:

- The identification of protein level changes in the three selected brain regions using 2D-DIGE and mass spectrometry analysis.
- Functional clustering of the detected changed proteins according to the UniProt and GeneOntology databases.
- Identification of potential protein regulators and targets with bioinformatics methods. Looking for overlaps with signal transduction members activated by hormones.
- Quantitative validation of some outstanding changed proteins with Western blot technique.
- Spatial distribution analysis of some outstanding changed proteins in the brain using histological techniques.

Materials and methods

We used 4 months old Wistar female rats in the experiments. The examined group was pup-caring, lactating mother rats and the control group was mothers deprived of their pups immediately after parturition. The hypothalamus, POA and mPFC regions were dissected on the 11^{th} *postpartum* day. We made the measurements on hypothalamic synaptosome samples and whole brain tissues from the POA and mPFC.

For the hypothalamic synaptosome samples, we used Saturation Labelling while Minimal Dye Labelling method was used for the whole brain POA and mPFC samples. Due to the limited protein concentration of hypothalamic samples we performed different types of labelling techniques. After the separation by charge and molecular weight of the proteins, the significantly changing protein spots were identified first on a preparative gel and after by a liquid chromatography system equipped Ultimate 3000 RSLC Nanosystem on the hypothalamic samples and by a LCQ Fleet ion trap mass spectrometry on the other samples.

The significantly changed proteins were principally classified by the available information from the UniProt (http://www.uniprot.org/) and GeneOntology (http://www.geneontology.org/) databases, based on their most relevant functions.

The linkage between the significantly changed proteins was analysed by Elsevier Pathway Studio Platform (version 2017). Our primary objective here was to find the potential common regulators and targets of the changed proteins.

We validated alterations of some outstanding proteins (C1qbp, Cryab) by Western blot analysis.

To establish if Cryab also changes at the mRNA level, RNA was isolated with TRIzol reagent from mPFC samples and subsequently reverse transcribed to cDNA. The cDNA products were used for qRT-PCR measurements in CFX96 Real-time System of Bio-Rad.

We analysed the spatial distribution of the C1qbp in the hypothalamus and the Cryab in the mPFC. We performed DAB immunolabelling on the hypothalamus slices. Double immunofluorescent labelling was also performed on the mPFC slices. The histological analysis was performed with an Olympus BX60 light microscope and with a Bio Rad 2100 Laser Scanning System equipped Nikon Eclipse E800 confocal microscope.

In situ hybridization histochemistry was used to establish the spatial distribution of the C1qbp RNA level in the hypothalamic region. First, probes were produced using amplified C1qbp cDNA product, which was sub-cloned into a TOPO TA vector containing T7 RNA polymerase recognition site. The T7 promoter was used to generate [^{35}S]UTP-labelled ribo-probes, with a MAXIscript transcription kit. Finally the tissues were prepared using an mRNA-locator kit. The development was made by Kodak developer fixed with Kodak fixer and counterstained with Giemsa and dehydrated and cover slipped with Cytoseal 60.

Immunocytochemical analysis of C1qbp protein in the synaptic area of the hypothalamus was carried out by post-embedding immunoassay. Before labelling the tissue was fixed, dehydrated and then was embedded in LR White. The ultrathin slides were also labelled on multi-step way and finally we acquired the images with a Morada CCD camera

equipped JEOL JEM 1011 electron microscope.

Results and theses

- We revealed protein changes in hypothalamic synaptosomes, and also whole tissue homogenates of the POA and mPFC regions in pup-caring lactating mother rats. We identified 26 significant protein changes in the hypothalamus, 18 in the POA and 32 in the mPFC.

- Using functional clustering we found that the largest portion of the altered proteins was in the group of electron transport chain and tricarboxylic acid cycle proteins in the hypothalamus, glucose metabolism in the POA, and synaptic and neuronal function in the mPFC. Interestingly, we found that most of the energy homeostasis related proteins showed decreasing amount in both the hypothalamus and POA. However, most of the synaptic and neuronal function related cortical proteins showed increased level.

- The protein changes were compared with previous gene expression experiments. We found 61% overlaps in the POA and 69% overlaps in the mPFC. These overlapping RNAs showed the same direction of changes as the protein changes. We did not find any overlaps between the hypothalamic protein changes and previous gene expression results.

- From the bioinformatics analysis, we revealed some common regulators and targets, which showed overlap with some signal transduction pathways activated by hormone receptors (oestrogen, progesterone, oxytocin and prolactin).

- One of the hypothalamic protein differences was the C1qbp, which is a multifunctional and mitochondrial protein. We validated decrease in the level of C1qbp with Western blot technique in lactating mother rats. We determined the localization of the protein in some hypothalamic neuronal synapses and also in the intracellular space. We carried out RNA level analysis with in situ hybridization histochemistry and found the same expression pattern of labelled neurons than with immunolabeling.

- In both the POA and mPFC samples we found Cryab as the highest changing protein. Its increase was also validated by Western blot technique. In the mPFC, we also demonstrated an increased amount of Cryab RNA level in lactating mother rats using qRT-PCR.

- The Cryab protein was found in the prelimbic and infralimbic cortical regions of the mPFC using immunohistochemistry. Double labelling revealed the presence of C1qbp in parvalbumin-containing local inhibitor neurons. In the calbindin-containing neurons, we did not observe the presence of Cryab.

Conclusions

We presented firstly the protein changes induced by the adaptation processes in lactating mothers. The results are derived from three brain regions established to control maternal behaviour. Previously, no regions of the brain had been examined by such an overall, exploratory proteomic analysis. Some of the identified proteins were presented in more than one protein spots, which imply posttranslational modifications (PTM). These PTMs also can be the final results of behavioural fine-tuning processes. During the interpretation of our results we must emphasize that the lack of lactation and the stress caused by pup deprivation could cause some of the differences. However, these processes disappear by the examined 11th *postpartum* day. Therefore, we believe that most of the protein differences between the two examined groups primarily derive from the differences in the maternal behaviour and lactation. Another important factor have to be considered through the evaluation is the very heterogeneous neuron populations in the examined regions, which could mask some of the smaller changes.

The interpretation of the changed proteins:

- Based on the results from hypothalamus and POA, it can be assumed that they were affected by an enhanced activation of the local neurons as leading to metabolic burden on the neurons as we found decrease in a large number of energy homeostatic proteins. It is known that enhanced activation of the neurons can lead to acidification, which finally can cause cell death. We presume a compensatory mechanism in proteins,

which can support some previous results (Vanoye-Carlo et al., 2009, Vergara-Castaneda et al.., 2016). We also found changes in the levels of protein implicated in synaptic function and neuronal development in the mPFC, the well-known centre of the cognitive abilities.

- Based on the comparison of our changes with some recently published gene expression experiments we identified significant similarities even though it is difficult to determine correlation between RNA and protein measurements (Greenbaum et al., 2003). Based on a higher than 60% overlaps in our case, we can speculate on similar trends of changes in the gene and protein expression levels. However, the ratio of the changes was more considerable in the protein levels than the RNA levels, which could reflect more frequent regulatory processes at the level of protein as opposed to RNA level.

- The large numbers of overlapping common regulators and targets of the proteins with the signal transduction pathways activated by hormonal receptors indicate some potential regulatory roles of hormones. However, a definite conclusion on the role of specific hormones cannot be drawn from these results because the highly overlap between the signal transduction pathways of the hormones do not allow us to differentiate the distinct hormonal effects.

- The translational regulation of the mitochondrial energy homeostasis proteins is one example of the multiple functions of C1qbp. However, the way how C1qbp level is regulated and how this protein affect other mitochondrial proteins has not been determined yet. Based on recently published results there are some correlations between the decreased protein amount of C1qbp and the decreased amount of some oxidative phosphorylation and tricarboxylic acid cycle related proteins (Yagi et al., 2012). Therefore, C1qbp may be a significant background factor behind our results. The subsequent histological analysis of C1qbp confirmed the presence of the protein in the examined brain region and also in the mitochondria in the subcellular level.

- The largest increase in both POA and mPFC regions was shown by Cryab, a small heat shock protein. It has been reported to increase in some neurological diseases

where its neuroprotective function was assumed (Rekas et al., 2004). In addition, the Cryab is present in two protein spots suggesting different PTMs. The presence of some of the PTMs can be correlated with the neuroprotective function of the protein (Benn et al., 2002). The parvalbumin-containing neurons, which we showed to express Cryab in the mPFC, coordinate the function of the pyramidal neurons. The dysfunction of this type of neurons can be associated with stress and depression (Sauer et al., 2015). Accordingly, to the presence of Cryab in parvalbumin-containing neurons can indicate protection of these neurons to ensure their optimal operation in the *postpartum* period.

The resulted data-based working hypotheses:

1. The parental care is distinct from other social behaviours because of the widespread synaptic and non-synaptic protein changes detected in the hub regions. These protein alterations can be caused by hormonal changes. Most of the changed proteins are mitochondrial, which suggest a new research direction: to examine metabolic alterations of the neurons in the *postpartum* period.
2. The multifunctional and chaperone like proteins (e.g. C1qbp, Cryab) can have some privileged roles in the presented molecular alterations. Thus, it would be important to reveal the neuronal interaction networks of the proteins and the effects of the hormonal system on them.
3. The identified altered proteins in correlation with parental care can be present in the CSF and in the serum, and consequently can serve as potential biomarkers. These biomarkers can be used for pharmaceutical research and/or for therapeutic purposes, e,g, for postpartum depression.

References

Benn, S. C., et al. (2002). "Hsp27 upregulation and phosphorylation is required for injured sensory and motor neuron survival." *Neuron* 36(1): 45-56.

Chen, P. and W. Hong (2018). "Neural Circuit Mechanisms of Social Behavior." *Neuron* 98(1): 16-30.

Dalley, J. W., et al. (2004). "Prefrontal executive and cognitive functions in rodents: neural and neurochemical substrates." *Neurosci Biobehav Rev* 28(7): 771-784.

Driessen, T. M., et al. (2014). "Genes showing altered expression in the medial preoptic area in the highly social maternal phenotype are related to autism and other disorders with social deficits." *BMC Neurosci* 15: 11.

Dulac, C., et al. (2014). "Neural control of maternal and paternal behaviors." *Science* 345(6198): 765-770.

Eisinger, B. E., et al. (2014). "Medial prefrontal cortex: genes linked to bipolar disorder and schizophrenia have altered expression in the highly social maternal phenotype." *Front Behav Neurosci* 8: 110.

Gammie, S. C., et al. (2016). "Genetic and neuroendocrine regulation of the postpartum brain." *Front Neuroendocrinol* 42: 1-17.

Goodson, J. L. (2005). "The vertebrate social behavior network: evolutionary themes and variations." *Horm Behav* 48(1): 11-22.

Greenbaum, D., et al. (2003). "Comparing protein abundance and mRNA expression levels on a genomic scale." *Genome Biol* 4(9): 117.

Rekas, A., et al. (2004). "Interaction of the molecular chaperone alphaB-crystallin with alpha-synuclein: effects on amyloid fibril formation and chaperone activity." *J Mol Biol* 340(5): 1167-1183.

Saper, C. B. and B. B. Lowell (2014). "The hypothalamus." *Curr Biol* 24(23): R1111-1116.

Sauer, J. F., et al. (2015). "Impaired fast-spiking interneuron function in a genetic mouse model of depression." *Elife* 4.

Vanoye-Carlo, A., et al. (2009). "Estrogen receptors increased expression during hippocampal neuroprotection in lactating rats." *J Steroid Biochem Mol Biol* 116(1-2): 1-7.

Vergara-Castaneda, E., et al. (2016). "Prolactin mediates neuroprotection against excitotoxicity in primary cell cultures of hippocampal neurons via its receptor." *Brain Res* 1636: 193-199.

Yagi, M., et al. (2012). "p32/gC1qR is indispensable for fetal development and mitochondrial translation: importance of its RNA-binding ability." *Nucleic Acids Res* 40(19): 9717-9737.

Publications related to the thesis:

1. **Udvari EB**, Völgyi K, Gulyássy P, Dimén D, Kis V, Barna J, Szabó ÉR, Lubec G, Juhász G, Kékesi KA, Dobolyi Á (2017) Synaptic proteome changes in the hypothalamus of mother rats. *Journal of Proteomics* 159:54-66.

2. Völgyi K, **Udvari EB**, Szabó ÉR, Györffy BA, Hunyadi-Gulyás É, Medzihradszky K, Juhász G, Kékesi KA, Dobolyi Á (2017) Maternal alterations in the proteome of the medial prefrontal cortex in rat. *Journal of Proteomics* 153:65-77.

3. **Udvari EB**, Völgyi K, Kékesi KA, Simon D, Hunyadi-Gulyás É, Dobolyi Á (2019) Proteomic analysis of the maternal preoptic area in rats. *Neurochemical Research* doi: 10.1007/s11064-019-02755-y

Publications unrelated to the thesis:

1. Völgyi K, Badics K, Sialana FJ, Gulyássy P, **Udvari EB**, Kis V, Drahos L, Lubec G, Kékesi KA, Juhász G (2018) Early presymptomatic changes in the proteome of mitochondria-associated membrane in the APP/PS1 mouse model of Alzheimer's Disease. *Molecular Neurobiology* 55(10): 7839-7857.

2. Barna J, Dimén D, Puska G, Kovács D, Csikós V, Oláh Sz, **Udvari EB**, Pál G, Dobolyi Á (2019) Complement component 1q subcomponent binding protein in the brain of the rat. *Scientific Reports* 9(1): 4597.